Analysis of Dishevelled signalling pathways during *Xenopus* development

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Background: Recent studies have demonstrated that the Wnt, Frizzled and Notch proteins are involved in a variety of developmental processes in fly, worm, frog and mouse embryos. The Dishevelled (Dsh) protein is required for *Drosophila* cells to respond to Wingless, Notch and Frizzled signals, but the molecular mechanisms of its action are not well understood. Using the ability of a mutant form of the *Xenopus* homologue of Dsh (Xdsh) to block Wnt and Dsh signalling in a model system, this work attempts to clarify the role of the endogenous Xdsh during the early stages of vertebrate development.

Results: A mutant Xdsh (Xdd1) with an internal deletion of the conserved PDZ/DHR domain was constructed. Overexpression of *Xdd1* mRNA in ventral blastomeres of *Xenopus* embryos strongly inhibited induction of secondary axes by the wild-type *Xdsh* and *Xwnt8* mRNAs, but did not affect the axis-inducing ability of β-catenin mRNA. These observations suggest that Xdd1 acts as a dominant-negative mutant. Dorsal expression of *Xdd1* caused severe posterior truncations in the injected embryos, whereas wild-type *Xdsh* suppressed this phenotype. *Xdd1* blocked convergent extension movements in ectodermal explants stimulated with mesoderm-inducing factors and in dorsal marginal zone explants, but did not affect mesoderm induction and differentiation.

Conclusions: A vertebrate homologue of Dsh is a necessary component of Wnt signal transduction and functions upstream of β-catenin. These findings also establish a requirement for the PDZ domain in signal transduction by Xdsh, and suggest that endogenous Xdsh controls morphogenetic movements in the embryo.

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Background

Wnts were first discovered as gene products involved in *Drosophila* embryo segmentation and in mammary tumorigenesis, and are now implicated in many developmental processes throughout the animal kingdom [1,2]. For example, Wnts have been shown to regulate tissue polarity in the *Caenorhabditis elegans* tail (the *lin-44* gene [3]), and development of the chicken and mouse limb [4–6], kidney [7,8] and somites [9]. Several Wnt products are thought to be essential for the proper development of different parts of the brain and spinal cord (for review, see [2]), and have been implicated in the establishment of dorsoventral and anteroposterior axes in vertebrates [10–13]. Despite increasing interest in Wnts, the molecular mechanisms of their signal transduction are largely unknown.

The Dishevelled (Dsh) and Armadillo (Arm) proteins are required for the response of *Drosophila* cells to Wingless (Wg) and were hypothesized to be components of the Wg signal transduction pathway [14]. The same signalling pathway appears to be conserved in vertebrates: *Xenopus* homologues of Dsh (Xdsh) and Arm (β-catenin and plakoglobin) and dominant-negative forms of glycogen synthase kinase 3β (GSK-3β; a negative regulator of the Wnt pathway) all mimic the ability of Wnts to induce a secondary body axis in *Xenopus* embryos [15–21]. Furthermore, the depletion of β-catenin in *Xenopus* oocytes results in dorsal-deficient development and in loss of responsiveness to a Wnt signal [22], suggesting that β-catenin is essential both for Wnt signalling and for axis formation.

The role of Dsh proteins in Wnt signalling is not clear. Xdsh [15] is a novel maternal protein consisting of three conserved domains which are present both in the fly Dsh [23,24] and in Dvl1, a mammalian homologue of Xdsh [25]. Whereas two of these domains are unique to Dsh homologues, the third, internal domain contains a PDZ domain (also known as the DHR or GLGF motif), which is present in the brain-specific protein PSD-95, the *Drosophila* tumor suppressor product Discs-large, the epithelial tight junction protein ZO-1, and in several other cytoskeleton- and membrane-associated proteins [26,27]. Dsh has been shown to be phosphorylated in response to Wg [28] and to interact with the Notch receptor both genetically and physically [29]. Overexpression of Xdsh can, on its own, mimic Wnt signalling, leading to the induction of a secondary body axis in *Xenopus* embryos [15]. Thus, Dsh appears to be functionally active in the absence of exogenously supplied Wnt

signals [15,16,28], but the mechanism by which Dsh functions remains to be studied.

To investigate the role of Dsh in vertebrate development, in particular in the establishment of the dorsoventral axis, two different mutated forms of Xdsh were constructed. One of these mutants (Xdd1), which lacked a portion of the PDZ domain, inhibited the ability of the wild-type Xdsh to trigger secondary axis formation. Consistent with the proposed role for Xdsh in Wnt signal transduction, Xdd1 abolished the axis-inducing activity of Xwnt8, but did not interfere with axis induction by β-catenin. The effect of the dorsally injected Xdd1 on normal development was unexpected: cell movements necessary for proper body-plan formation at the neurula stages were strongly inhibited, whereas head development and dorsal mesoderm differentiation were not affected in the injected embryos. These results establish the requirement for Xdsh in Wnt signal transduction and in the control of morphogenetic cell movements.

Results

Xdd1 inhibits the formation of secondary axes induced by Xdsh and Xwnt8, but not those induced by β-catenin

The ability of mutated Xdsh products with deletions in the coding region of Xdsh cDNA (see Materials and methods) to interfere with Xdsh function was studied. A form of Xdsh with an internal deletion of a portion of the PDZ domain (Xdd1) strongly inhibited the axis-inducing ability of wild-type *Xdsh* mRNA upon co-injection into a ventral blastomere of the four-cell embryo (Table 1). Whereas *Xdsh* mRNA induced complete secondary axes in 83 % of injected embryos ($n = 81$), when Xdsh was injected simultaneously with *Xdd1* mRNA, the induction of complete axes was reduced to only 6 % of embryos ($n = 62$). In contrast, injections of a control *Xdd2* mRNA, encoding another Xdsh mutant with a carboxy-terminal deletion, had no effect on the axis-inducing ability of Xdsh. On its own, *Xdd1* mRNA failed to induce secondary axes when overexpressed in ventral blastomeres of four-cell embryos (Table 1; $n = 77$). Thus, Xdd1 behaved in a dominant-negative manner and was chosen for further studies.

In *Drosophila* embryos, Dsh is required for reception and transduction of Wg signals [14]. If the Wnt signalling pathway is conserved between *Drosophila* and vertebrates, vertebrate homologues of Dsh should retain their essential role in transmission of Wnt signals, and a dominant-negative form of Xdsh might be expected to block Wnt signalling. As several Wnts (including Wnt1, Xwnt8 and Xwnt3a) have been shown to induce secondary axes upon microinjection of their mRNAs into ventral blastomeres of early *Xenopus* embryos [2,10,11], the ability of *Xdd1* mRNA to interfere with this axis induction was assessed.

Xwnt8 mRNA was microinjected into a ventral blastomere of four-cell embryos with or without *Xdd1* RNA. The majority of embryos injected with 2 pg of *Xwnt8* mRNA developed secondary axes (92 %, Table 1; Fig. 1a) as judged by the appearance of a second cement gland and a second set of eyes. These embryos were shown previously to have a complete duplication of dorsal structures including notochord, neural tube and somites [10]. Secondary axis induction was completely suppressed when injections of *Xwnt8* mRNA were accompanied by microinjection of 1.5 ng of *Xdd1* mRNA (Table 1; Fig. 1b). In contrast, *Xdd2* mRNA failed to interfere with the axis-inducing ability of *Xwnt8* mRNA (data not shown). A supplementary injection of the wild-type *Xdsh* mRNA together with *Xwnt8* and *Xdd1* mRNAs restored duplication of axial structures in 72 % of injected embryos ($n = 28$), further arguing against

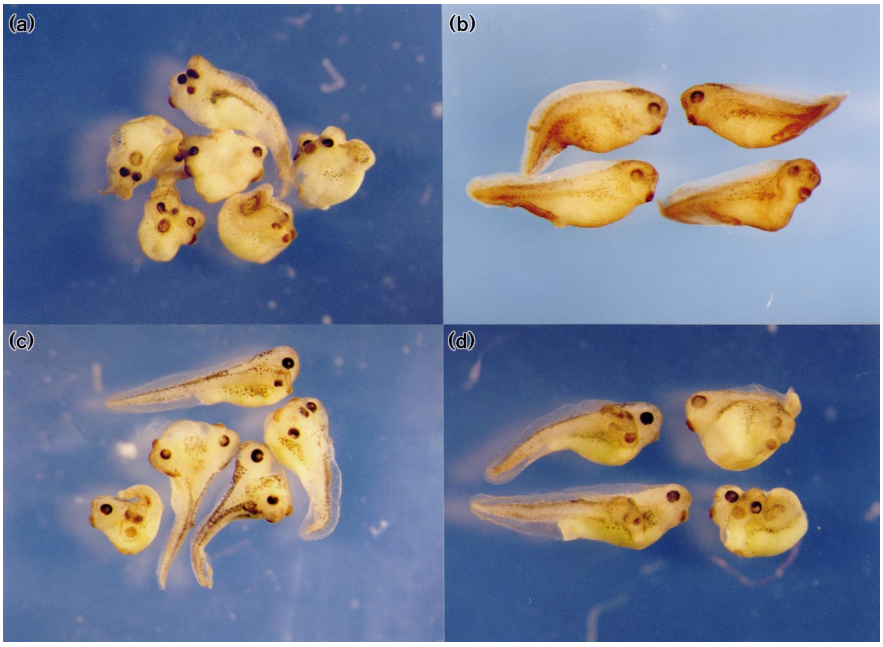
Table 1

Effect of *Xdd1* mRNA on induction of secondary body axes by *Xwnt8*, *Xdsh* and *Xβcat* mRNAs.

mRNA injected	Number of embryos injected	Embryos with a complete second axis	Embryos with a partial second axis	Normal body axis	Other developmental defects
Xdsh	81	67	4	8	2
Xdd1	77	0	2	72	3
Xdd2	46	0	0	45	1
Xdsh + *Xdd1*	62	4	12	44	2
Xdsh + *Xdd2*	27	21	2	4	1
Xwnt8	39	36	1	2	0
Xwnt8 + *Xdd1*	42	0	2	38	3
Xβcat (1 ng)	84	73	5	4	2
Xβcat (1 ng) + *Xdd1*	82	71	4	1	6
Xβcat (0.2 ng)	41	6	32	3	0
Xβcat (0.2 ng) + *Xdd1*	46	4	39	1	2

Synthetic mRNAs were injected into a single ventral blastomere of four- to eight-cell stage embryos. Doses of mRNA per embryo were 1.5 ng for *Xdsh*, *Xdd1* or *Xdd2*, 2 pg for *Xwnt8* and 0.2 ng or 1 ng for β-catenin mRNA (*Xβcat*). Induction of a second body axis was scored by the appearance of a second neural plate on the ventral side of early neurulae and ectopic eyes and cement glands at the tadpole stages (complete second axis) or ectopic secondary neural plate lacking anterior most structures (partial body axis). Other defects included failure of the blastopore to close, microcephaly and tail truncations. The data are combined from four independent experiments.

Figure 1

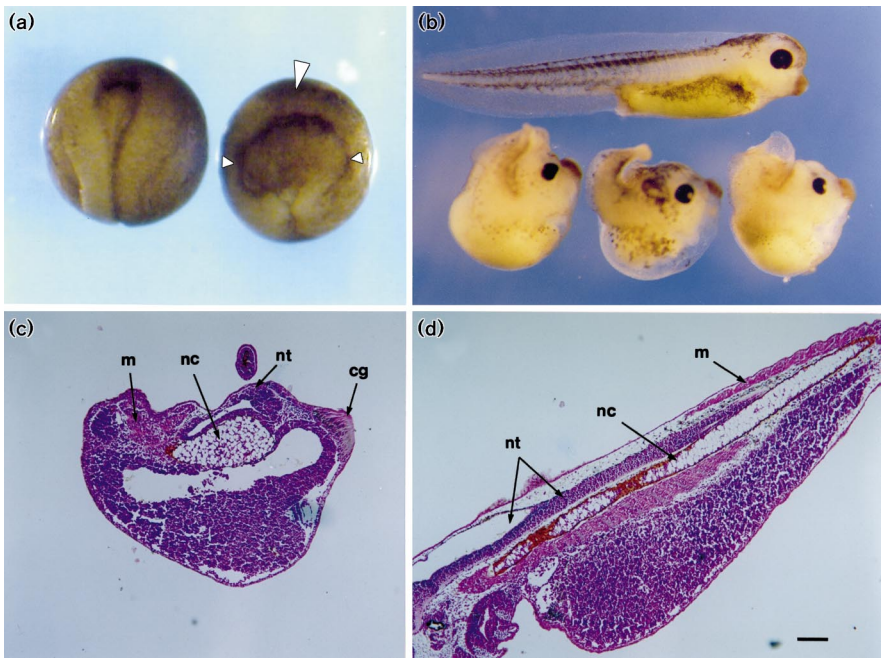


The effect of *Xdd1* mRNA on axis-inducing ability of *Xwnt8* and *Xβcat* mRNAs. One ventro-vegetal blastomere of 4–8-cell embryos was injected with **(a)** 2 pg of *Xwnt8* mRNA, **(b)** 2 pg of *Xwnt8* mRNA plus 1.5 ng of *Xdd1* mRNA, **(c)** 0.8 ng of *Xβcat* mRNA or **(d)** 0.8 ng of *Xβcat* mRNA plus 1.5 ng of *Xdd1* mRNA.

nonspecific inhibitory effects of Xdd1. These findings suggest that the Xdsh function is essential for induction of secondary axes by Wnts.

In the same series of experiments, *Xdd1* RNA failed to interfere with axial structures induced by 1 ng of β-catenin mRNA (Table 1; Fig. 1c,d) upon ventral blastomere injection. β-catenin mRNA was then diluted until its axis-inducing activity became much weaker. At a dose of 250 pg, β-catenin mRNA induced only partial secondary axes. Even at this low dose, the effect of β-catenin mRNA was not inhibited by 1 ng of *Xdd1* mRNA (Table 1), whereas the same dose of *Xdd1* mRNA readily blocked the majority of axes induced by Xwnt8. This result is consistent with epistatic genetic analysis in *Drosophila* embryos indicating that Dsh functions upstream of Arm [14].

Figure 2



Embryos microinjected with *Xdd1* mRNA develop a specific morphogenetic defect. Two-cell embryos were injected dorsally with 1 ng of each mRNA into each blastomere. **(a)** Stage 14 neurulae injected with *Xdd1* mRNA (on the right) and with *Xdd2* mRNA (on the left). Note the failure of neural folds (shown by two small white arrowheads) to close into a neural tube. The cement gland develops at the anterior end (large arrowhead); the closed blastopore is visible at the posterior end. The embryo injected with *Xdd2* mRNA has a normal neural tube and is indistinguishable from uninjected embryos and from embryos injected with wild-type *Xdsh* (not shown). **(b)** Stage 40 tadpoles. Top: control embryo (uninjected); bottom: *Xdd1*-injected embryos with posterior deficiencies. **(c)** Sagittal histological section of *Xdd1*-injected embryo (stage 40); **(d)** sagittal section of a control embryo (stage 40). Abbreviations: m, muscle; nc, notochord; nt, neural tissue; cg, cement gland. The bar in (d), which also refers to (c), is 200 μm.

Table 2

Effect of *Xdd1* mRNA on normal embryogenesis.

mRNA injected	Injection site	Stage (number of cells)	Number of embryos injected	Normal body axis	Xdd1 phenotype	Other defects*
Xdd1	Dorsal equatorial	2–4	107	3	92	12
Xdd2	Dorsal equatorial	2–4	59	56	1	3
Xdsh	Dorsal equatorial	2–4	49	40	5	4
Xdsh + *Xdd1*	Dorsal equatorial	2–4	99	57	31	11
Xdd1	Ventral equatorial	2–4	56	53	2	1
Xdd1	Dorsal animal	16–32	41	3	34	4
Xdd1	Dorsal vegetal	16–32	44	32	5	7

Synthetic mRNAs (0.5–1 ng each) were injected symmetrically into two blastomeres as shown, and their effect on embryogenesis ('Xdd1 phenotype') was scored morphologically for open neural plate at stages 16–17 and for specific trunk/tail truncation (see Fig. 2) at stages 35–40. The data are combined from several independent experiments. *Other defects included failure of the blastopore to close, microcephaly and kinked tails.

Together, these experiments indicate that Xdsh is required for Wnt signalling during secondary axis formation in *Xenopus* embryos and that it functions upstream or parallel to β-catenin in the Wnt signal transduction pathway in vertebrates.

Effects of the Xdd1 mutant on morphogenesis

Although the above observations establish the requirement for Xdsh in the Wnt-induced secondary axis formation, they do not define the role of Xdsh in normal development. As Wnt signalling has been implicated in dorsal development, blocking endogenous Xdsh function might be expected to inhibit formation of 'Spemann's organizer', a dorsal signalling center [30], and to give rise to 'ventralized' embryos which are deficient in anterior and dorsal structures [30,31].

To investigate the function of endogenous Xdsh in *Xenopus* embryos, *Xdd1* mRNA was introduced into the dorsal marginal region of two-cell embryos and their development was monitored closely. The development of embryos injected with 1 ng of *Xdd1* mRNA into both blastomeres proceeded with no visible deviations from that of uninjected controls until blastopore closure at the end of gastrulation. Later, however, the neural plate of the injected embryos remained wide open, and the neural tube failed to close (Fig. 2a). At later stages, these embryos had a dramatically shortened body axis and dorsally bent tails (Fig. 2b–d; Table 2). The same phenotype was observed when *Xdd1* mRNA was injected dorsally into the fertilized egg before the first cleavage (data not shown). Despite posterior truncations and the virtual absence of a tail in severe cases, *Xdd1*-injected embryos formed morphologically normal head structures with well-defined eyes and cement glands (Fig. 2a–c). These unexpected findings argue against the involvement of Xdsh in dorsoventral axis determination, because inhibition of dorsal development by ultraviolet light or by surgical extirpation of the organizer leads primarily to anterior deficiencies [31,32].

Shortening of the anteroposterior axis is likely to arise through inhibition of the morphogenetic movements necessary for body elongation (for review, see [33]). This novel developmental phenotype was observed in 90 % of injected embryos (Table 2) and appears to be a specific result of *Xdd1* mRNA overexpression.

The effect of *Xdd1* was dose-dependent. At higher doses of *Xdd1* mRNA (2–4 ng), eyes failed to develop at high frequency, whereas at lower doses (0.1–0.3 ng), the phenotype became less pronounced: the injected embryos developed essentially normally, with the exception of a kinked head (see Discussion). Injections into different blastomeres at the 32-cell stage indicated that dorso-animal blastomeres were more sensitive to *Xdd1* than dorso-vegetal blastomeres (Table 2). Control injections of

Figure 3

Wild-type *Xdsh* mRNA corrects dorso-posterior deficiencies caused by injections of *Xdd1* mRNA. Injections of wild-type *Xdsh* (top three embryos), *Xdd1* (middle row) and a mixture of both *Xdd1* and wild-type *Xdsh* (bottom row) were performed as described in Figure 2. A control uninjected embryo is shown on the left.

1 ng of *Xdd2* or wild-type *Xdsh* mRNAs did not affect normal development significantly (Table 2). Occasionally, wild-type *Xdsh* mRNA injections resulted in the formation of a wider notochord, probably reflecting the ability of Xdsh to induce an ectopic body axis (see also [34]).

These findings suggest that Xdsh is required for the control of morphogenetic cell movements after gastrulation [33] and fail to demonstrate a role for Xdsh in dorsoventral axis determination.

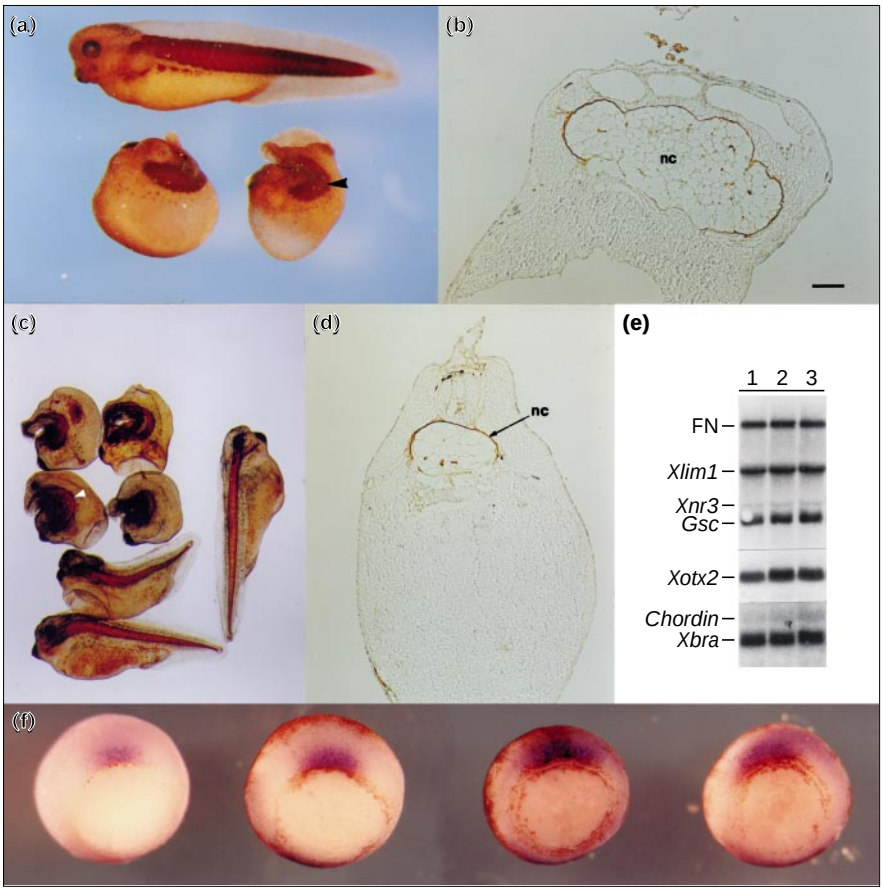
Rescue of Xdd1-induced defects by expression of wild-type Xdsh

As the Dsh protein lacks any known enzymatic activities, it may function as an 'adaptor' protein that brings different proteins into close apposition, allowing them to interact during signal transduction (see Discussion). The ability to bind the endogenous regulators of Xdsh without being able to transduce a signal to downstream components of the pathway (for example, β-catenin) may provide a possible explanation for the effects of Xdd1. If the Xdd1 phenotype is due to specific dominant-negative interference, *Xdd1*-injected embryos should be rescuable by co-injection of the full-length *Xdsh* mRNA. In agreement with this expectation, the wild-type *Xdsh* mRNA significantly suppressed morphogenetic defects caused by *Xdd1* after injections into the dorsal margin of embryos at the two- to four-cell stage (Fig. 3, Table 2). In contrast to the effect of *Xdsh* mRNA, β-galactosidase (*β-gal*) and *Xdd2* mRNA failed to rescue *Xdd1*-induced deficiencies, further arguing for specificity of the Xdd1 phenotype (data not shown). These results argue against Xdd1 sequestering a critical cell component in a nonspecific manner. As Xdd1 and Xdsh differ only in the PDZ domain, this region appears to be essential for Xdsh signalling in early embryos. Together, these and other [28] findings point to the essential role of the PDZ domain in transmission of Wnt signals inside the cell and during *Xenopus* embryogenesis.

Spemann organizer markers and mesodermal cell fates are not affected by Xdd1

To determine which developmental process is affected by the dominant-negative form of Xdsh, two-cell embryos that had been injected with *Xdd1* dorsally into both blastomeres were analyzed in more detail (Figs 2c,d and Fig. 4). Histological sections revealed the presence of abundant notochord and malformed neural tissue (Figs 2c and 4b).

Figure 4



Early marginal zone markers and mesodermal tissue differentiation are not affected by *Xdd1*. (**a**) Whole-mount immunochemical staining with muscle-specific 12/101 antibodies. Top: control embryo; bottom: two embryos injected dorsally with *Xdd1* mRNA. Arrowhead points to a somite. (**b**) Cross-section of an *Xdd1*-injected embryo after whole-mount staining with notochord-specific MZ15 antibodies. Brown staining outlines a sheath around notochord (nc). Bar in (b), which also refers to (d), is 150 μm. (**c**) Whole-mount staining with MZ15 antibodies. Top left: four embryos injected with *Xdd1* mRNA; white arrowhead points to notochord. Bottom left: two embryos injected with wild-type *Xdsh* mRNA; control uninjected embryo is shown on the right. (**d**) Cross-section of a control uninjected embryo stained with MZ15 antibodies. (**e**) Northern analysis of early marginal zone markers in control embryos (lane 1) and in embryos injected dorsally with *Xdd1* (lane 2) or *Xdsh* (lane 3) mRNAs. Total RNA was prepared when control embryos reached stage 10.5. *Xbra* is a general marginal zone marker. *Xotx2*, *goosecoid* (*Gsc*), *Xlim1* and *chordin* are dorsal marginal zone markers. *Xnr3* is expressed in the superficial layer of the Spemann organizer. Fibronectin (FN) mRNA is a control for loading. (**f**) *Xdd1* does not alter the localization of chordin transcripts. *In situ* hybridization of stage 10.5 gastrula embryos with *chordin* anti-sense RNA probe. Far left: uninjected albino embryo; centre left: uninjected pigmented embryo; right: two pigmented embryos injected with *Xdd1* mRNA in the dorsal margin at the two- to four-cell stage.

To further compare mesodermal tissues in *Xdd1*-injected and control embryos, whole-mount immunohistochemistry was carried out. Staining with the notochord-specific monoclonal antibodies MZ15 showed that well-differentiated notochordal tissue was present in both control and *Xdd1*-injected embryos (Fig. 4c). Although the notochord was often expanded in width, it was much shorter in *Xdd1*-injected embryos than in control embryos (Figs 2c and 4c), suggesting that Xdd1 affects the elongation and final shape of the notochord, but not its differentiation. Likewise, staining with the muscle-specific 12/101 antibodies showed that *Xdd1*-injected embryos contained well-differentiated but shortened ('condensed') somites (Fig. 4a). These observations indicate that Xdd1 does not affect dorsal mesoderm induction and differentiation.

Morphogenetic movements that lead to the establishment of the basic body plan are controlled by the Spemann organizer [30,31,33]. Although mesodermal tissues appear to differentiate normally in *Xdd1*-injected embryos, early specification of the Spemann organizer may be altered by *Xdd1* mRNA. To assess this possibility, total RNA from injected embryos was extracted for northern analysis of organizer (dorsal marginal zone) markers at the early gastrula stage. The expression of several marginal zone markers including *Xbra*, *goosecoid*, *Xlim1*, *Xnr3*, *Xotx2* and *chordin* was not affected significantly by *Xdd1* mRNA injection (Fig. 4e). To assess whether these markers remain expressed at the same location in the embryo, *in situ* hybridization was carried out for *chordin*, *Xlim1* and *goosecoid*. The localization of *chordin* transcripts (Fig. 4f) as well as those of *Xlim1* and *goosecoid* (data not shown) was not altered in *Xdd1*-injected embryos. Together, these observations indicate that Xdd1 does not affect Spemann organizer markers and suggest that the abnormal embryonic phenotype does not result from the effect of Xdd1 on dorsal mesoderm formation.

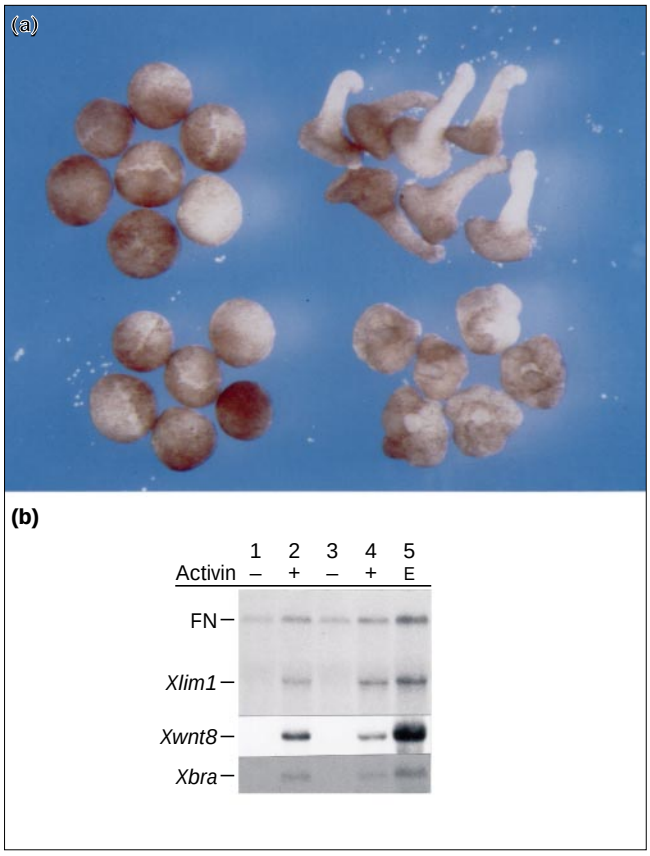
Xdsh is required for morphogenetic movements of animal caps stimulated with mesoderm-inducing factors

To further clarify the basis for the developmental abnormalities of *Xdd1*-injected embryos, the induction of mesoderm in presumptive ectodermal explants (animal caps) was analyzed. When cultured in isolation, animal caps differentiate into atypical epidermis [30]. Upon treatment with a variety of developmental signalling factors, animal caps form mesodermal and neural tissues. The mesoderm-inducing factors activin and fibroblast growth factor (FGF) cause animal caps to undergo extensive cell rearrangements known as convergent extension movements [33], which are coincident with mesoderm induction [35].

Injection of 1 ng of *Xdd1* mRNA into each animal hemisphere at the two-cell stage strongly inhibited activin-induced elongation of animal caps, whereas injection of the same amount of *Xdd2* mRNA had no effect (Fig. 5a). Despite the pronounced inhibition of explant elongation, *Xdd1* did not have a significant effect on the induction of early mesodermal markers *Xbra*, *Xwnt8* and *Xlim1* in induced animal caps (Fig. 5b).

The inhibition of animal cap cell movements by Xdd1 in response to activin (Fig. 5) and FGF (data not shown) is consistent with its effect on elongation of the whole embryonic body axis (Fig. 2). Together, these experiments suggest that the endogenous Xdsh protein is involved in the control of normal morphogenetic movements occurring at the gastrula and neurula stages of development.

Figure 5



Xdd1 inhibits morphogenetic movements of ectodermal explants stimulated with activin. **(a)** Morphology of explants at stage 17. Top left: control uninduced explants; top right: control explants induced with activin; bottom left: explants overexpressing *Xdd1*; bottom right: explants overexpressing *Xdd1* and stimulated with activin. No difference is apparent between control uninjected explants and explants injected with *Xdd2* (data not shown). **(b)** Northern analysis of early markers at the early gastrula stage (stage 10.5). Lanes 1,2: uninjected explants; lanes 3,4: *Xdd1*-injected explants; lane 5, control embryos. Treatment of explants with activin was as indicated. Total RNA was prepared when control embryos reached stage 10.5. *Xbra* is a general marginal zone marker. *Xlim1* marks dorsal, and *Xwnt8* marks ventrolateral marginal zone. Fibronectin (FN) mRNA is a loading control.

observations suggested that the Wnt pathway is conserved in both flies and in amphibians, but did not establish the requirement or role for Xdsh in Wnt signalling in vertebrates. The data presented in this study show that Xdsh is essential for Wnt-mediated ectopic induction of embryonic body axes (Fig. 7a).

Do Wnts play a role in dorsoventral axis determination?

The ability of Wnts and other members of the Wnt/Wg signalling pathway to induce a complete body axis in *Xenopus* embryos suggests that some endogenous Wnt(s) function to establish dorsoventral differences in the early embryo. Thus, blocking Wnt or Xdsh signalling might be expected to interfere with the normal process of dorsoventral axis determination and Spemann organizer formation. Contrary to these expectations, early transcriptional markers for dorsal mesoderm are induced normally, and head development is not significantly perturbed, in *Xdd1*-injected embryos, indicating that Xdd1 does not interfere with the early determination of the dorso-ventral axis in *Xenopus* (Figs 2a,b and 4e). This observation contrasts with the ability of GSK-3, a negative regulator of Wnt/Wg signal transduction, to inhibit the organizer-specific expression of *goosecoid* [20] and to cause microcephaly [19], and with the earlier findings that depletion of the endogenous β-catenin mRNA with antisense oligonucleotides leads to axial deficiencies [22].

Several possible explanations can be put forward to account for these results. First, a normal developmental program — other than Wnt(s) — may trigger signal transduction pathway at some step downstream of Dsh. For example, cortical rotation, which has been tightly linked to establishment of dorsoventral differences in the fertilized egg [31], may lead directly to downregulation of GSK-3 activity or to stimulation of β-catenin. In this case, Wnt or Dsh signalling becomes dispensable for dorsoventral axis formation, but can induce secondary axes upon ventral injection.

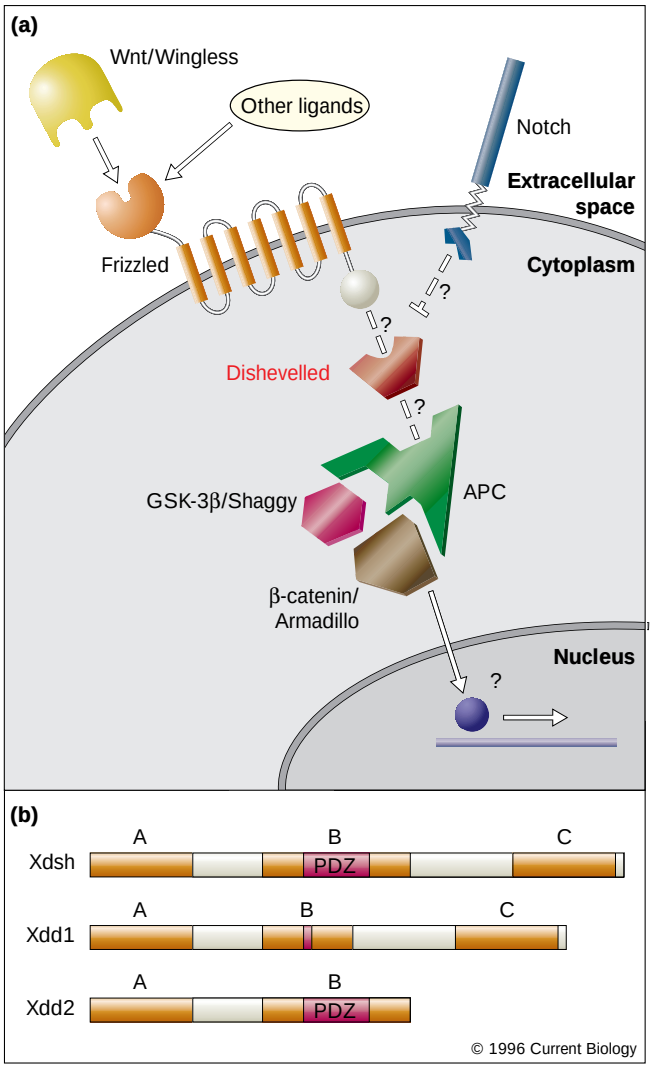
Second, an effect of Xdd1 on dorsal axis formation may not be observed because Xdd1 protein does not accumulate to a level that is sufficient to block early axis formation. This explanation is not likely, because many *Xdd1* mRNA injections were performed before the first cleavage, whereas *gsk3* mRNA has been reported to inhibit dorsal development when injected at the four-cell stage [19,20]. These observations suggest that the critical time for dorsoventral axis determination is at least as late as the four-cell stage.

Finally, blocking Dsh signalling may not necessarily abolish all Wnt signals. For example, Wg autoregulation in *Drosophila* does not appear to require Dsh function [36]. Conversely, *Drosophila* Dsh is known to participate in signal transduction by the Notch or Frizzled pathways, which may not necessarily depend on Wnts [37,29]. Thus, Dsh-mediated signal transduction should not be considered synonymous with Wg/Wnt signalling, and the role of Wnts in the establishment of the dorsoventral axis remains to be clarified by future studies.

The role of the endogenous Xdsh in morphogenesis

The developmental phenotype resulting from overexpression of *Xdd1* in the dorsal marginal zone indicates that the endogenous Xdsh product controls stretching of tissues during morphogenesis. Several types of cell behavior have been described for early embryogenesis (see [33]). Morphogenetic movements that mainly involve thinning of the

Figure 7



(a) Interaction of Dishevelled with other components of the Wnt signal transduction pathway. Dishevelled is thought to modulate the activity of β-catenin/Armadillo by transducing a signal from the Frizzled/Notch receptors to the GSK3/APC/β-catenin complex. This modulation leads to subsequent transcriptional activation of the target genes. **(b)** The wild-type and mutated Xdsh constructs used in this study.

Figure 6

The effect of *Xdd1* on cell movements may depend on cell–cell interactions. **(a)** *Xdd1* inhibits morphogenetic movements of the dorsal marginal zone. Dorsal marginal zone explants were isolated at stage 10 from embryos that had been injected earlier with 1 ng of *Xdd1* mRNA (on the left) or *Xdd2* mRNA (on the right). **(b,c)** Lineage tracing of *Xdd1*-microinjected embryos. (b) Embryos were injected at eight-cell stage into the dorso-animal blastomere with *β-gal* mRNA only (bottom embryo) or with *β-gal* mRNA plus *Xdd1* mRNA (two top embryos). (c) Two top dorso-animal blastomeres at the 32-cell stage were injected with 5 nl of *β-gal* RNA (two top embryos) or *Xdd1* plus *β-gal* RNAs (three bottom embryos). The arrowhead points to the area in which developmental abnormalities are observed, despite the absence of *Xdd1* and *β-gal* RNA (unstained). Embryos were cultured until stage 35, then fixed and β-gal-expressing cells were visualized histochemically.

Control of convergent extension by Xdsh requires cell–cell interactions

Although Xdd1 inhibited convergent extension in induced animal caps, it was not clear whether this is the mechanism by which Xdd1 causes posterior deficiencies in injected embryos. Animal pole cells represent a useful model system for studying mesoderm formation, but these cells do not normally participate in convergent extension movements that lead to body-axis elongation *in vivo*. To directly assess whether Xdd1 altered morphogenetic movements in the embryo, dorsal marginal zone explants were isolated from uninjected embryos and from embryos that were injected with *Xdd1* mRNA into the dorsal margin. When control embryos reached stage 17, elongation of *Xdd1*-injected explants was inhibited strongly compared with uninjected explants (Fig. 6a). This finding indicates that Xdd1-mediated inhibition of convergent extension movements in the embryo is a likely cause of the observed developmental abnormalities.

To evaluate whether *Xdd1* affects only those cells in which it is expressed, *β-gal* mRNA was co-injected with *Xdd1* mRNA as a lineage tracer into dorsal animal region of eight-cell embryos. These injections supplied gene products mainly to the prospective anterior neural tissues, because only head structures were stained for β-galactosidase activity in the developed tadpoles (Fig. 6b). Under these conditions, *Xdd1*-injected embryos ($n = 45$) developed the same developmental abnormalities as embryos injected in the dorsal marginal zone at the two-cell stage ($n = 29$; Fig. 6b). As injections at the eight-cell stage do not provide high resolution fate mapping, lineage tracing was also carried out for 32-cell stage embryos. The majority of embryos co-injected with *Xdd1* and *β-gal* RNAs into two top dorso-animal blastomeres ($n = 41$), had identical developmental phenotypes to those of embryos injected at the two-cell stage, and revealed β-galactosidase staining only in anterior ectodermal tissues (Fig. 6c). These unexpected findings indicate that morphogenetic movements are controlled by cell–cell interactions between the prospective head region and marginal zone cells. Consistent with this observation, the majority of embryos that were injected with *Xdd1* mRNA in a dorso-vegetal region developed normally (see Table 2). These findings suggest that the effect of Xdd1 is non-cell-autonomous and implies that the endogenous Xdsh is involved in a signal relay system that initiates and maintains morphogenetic movements in the embryo.

Discussion

Xdsh is required for Wnt signalling during secondary axis formation

It is shown here that Xdd1, a mutated form of Xdsh that has an internal deletion of the PDZ domain, behaves as a dominant-negative mutant by inhibiting wild-type Xdsh activity in co-injection experiments. Xdd1 also blocks Wnt-mediated induction of secondary axes and strongly suppresses convergent extension movements in *Xenopus* explants and embryos.

Although genetic studies in *Drosophila* have shown that the fly Dsh product should be present in cells responding to Wg, the requirement for homologues of Dsh in Wnt signal transduction in vertebrates has been supported only indirectly. Overexpression of Xdsh, as well as plakoglobin and β-catenin, is sufficient to trigger dorsal axis formation in *Xenopus* [15,17,18]. Dominant-negative forms of GSK-3, a homologue of Shaggy [19–21], have similar axis-inducing ability, consistent with the proposed negative-regulatory role of Shaggy in Wg signalling [14]. Together, these

blastocoel roof and expansion of presumptive ectodermal territory before and during gastrulation are known as epiboly. The main driving force for axis elongation is attributed to another type of cell rearrangements called convergent extension. Convergent extension represents a series of radial and mediolateral cell intercalations that generate an elongated array of cells destined to form notochord, somites and spinal cord [33]. Presumptive mesoderm is the primary germ layer undergoing this type of cell rearrangements, which begin at the early gastrula stage and continue through neurulation and tailbud elongation.

Xdd1 appears to inhibit only one specific type of morphogenetic movements. As the blastopore is closed normally in *Xdd1*-injected embryos, both animal pole epiboly and involution of the marginal zone during gastrulation seem not to be affected by injection of *Xdd1* mRNA. This effect of *Xdd1* is different from that of a dominant-negative FGF receptor which inhibits tissue involution across the blastopore lip and prevents the blastopore from closing [38]. Although both *Xdd1* and the dominant-negative FGF receptor cause posterior deficiencies, the developmental phenotypes of the injected embryos are quite distinct, suggesting that different types of morphogenetic movement are affected.

Convergent extension movements are brought about by inductive interactions which usually correlate with mesodermal cell-fate determination [35]. Consistent with this notion, Goosecoid, a homeodomain-containing protein, was shown to affect cell movements during gastrulation, presumably because of its ability to respecify mesodermal cell fates [39]. Similarly, a dominant-negative FGF receptor inhibits both convergent extension movements and induction of some early mesodermal markers, including *Xbra*, a homologue of mouse *brachyury*/*T* gene [38]. Furthermore, heparan sulfate proteoglycans are required for gastrulation movements because of their role in FGF-dependent cell fate specification [40]. Thus, several known factors affecting cell movements appear to have this effect by controlling mesodermal cell fates. In contrast to these examples, the effects of the Xdd1 mutant on morphogenesis do not seem to be accompanied by changes in mesodermal cell fates.

As β-catenin, which appears to act in the same signal transduction pathway but downstream of Dsh, is known to interact with cadherins, important regulators of cell adhesion, Xdd1 may inhibit morphogenetic movements through β-catenin-dependent cell adhesion (for example, see [22,41]). This idea seems less attractive considering that the effect of Xdd1 requires cell–cell interactions (Fig. 6b,c). The molecular nature of the Xdsh-mediated cell–cell interactions that are necessary for normal morphogenesis is not clear. Further studies are necessary to determine whether Xdsh affects cell adhesion, and to define the processes involving different types of convergent extension in the embryo.

How does Xdsh function?

Little is known about the molecular mechanism of Dsh function. *Drosophila* Dsh was reported to be phosphorylated in response to Wg, and this phosphorylation seems to be critical for its function [28]. Dsh has also been shown to bind to the Notch receptor and proposed to negatively regulate the Notch pathway [29].

The results of this study indicate that the PDZ domain is a critical regulator of Dsh function. PDZ/DHR domains are found in a variety of cytoskeletal and membrane-associated proteins [26,27]. The human Discs-large protein binds *via* its PDZ domain to protein 4.1, which links cytoskeletal elements to the cell membrane [42]. PDZ domains have also been shown to regulate binding of PSD-95, a membrane-associated guanylate kinase, to Shaker, a voltage-gated K^+ channel [43], and to mediate interactions of nitric oxide synthase with syntrophins [44]. Thus, PDZ domains may play a role in the control of protein–protein interactions adjacent to cell membranes and in the formation of macromolecular signal transduction complexes.

As it has no known enzymatic activities, Dsh may function by bringing two or more signal transduction components into close proximity to allow them to interact. If this is the case, one possible explanation for the present findings is that Xdd1 sequesters upstream regulatory elements of the Wnt pathway, and that the endogenous role for the PDZ domain is to bind to and activate downstream signalling components of the pathway. The implication of Frizzled-related proteins in the reception of Wnt/Wg signals [45], and the identification of complexes of adenomatous polyposis coli (APC) with GSK-3, β-catenin and Discs-large [46–47], allows one to build working models for how Wnt signals may be transduced in the cell (Fig. 7a). Further structural-functional studies are necessary to test such models and to determine the molecular basis for Xdsh activities.

The developmental phenotype of *Xdd1*-injected embryos is very similar to the effect of several Wnts (*Xwnt5a*, *Xwnt4* and *Xwnt11*), which are not capable of inducing a complete secondary axis on their own [41,48]. In both cases, morphogenetic movements of animal caps stimulated by activin are inhibited, but mesoderm differentiation in the explants is not affected. Furthermore, in whole embryos, injection of the low doses of *Xdd1* leads to formation of a kinked head and shortened body axis, thereby mimicking the effect of *Xwnt5a* mRNA injections [41,45]. This similarity of phenotypes suggests that Xwnt5a and related Wnts represent naturally occurring dominant-negative forms of Wnt1 or Xwnt8. These gene products may exert their effects by competing with Wnt1 or Xwnt8 for the same receptor, leading to local inhibition of Dsh-mediated signal transduction. Consistent with this hypothesis, *Wnt5a* mRNA has been recently reported to suppress axis-inducing activity of

Xwnt8 mRNA [49]. Evidence is accumulating to support the existence of similarly organized developmental regulators with mutually antagonizing activities. For example, Argos has been shown to compete with Spitz for binding with the *Drosophila* epidermal growth factor (EGF) receptor [50]; and Serrate may antagonize the effects of Delta by interacting with the same Notch receptor [51]. Similarly, ADAMP, a ventralizing TGFβ, and nodal-related TGFβs with dorsalizing properties, are co-expressed in the Spemann organizer [52,53]. These counteracting positive and negative influences could be used to establish tissue boundaries and may, therefore, represent a common patterning mechanism.

Conclusions

A mutated form of Xdsh lacking the PDZ domain blocks signalling by the wild-type Xdsh and by Xwnt8 in a dominant-negative manner. Injections of the mutant *Xdd1* mRNA show that Xdsh is an essential component of the signal transduction pathway operating during Wnt-mediated secondary body axis induction. Unexpectedly, dorsal microinjections of *Xdd1* mRNA failed to reveal a role for endogenous Wnts in dorsoventral axis determination; there was a strong inhibition of convergent extension movements in the embryo, leading to severe posterior truncations. This effect is not likely to be due to a nonspecific sequestration of some critical cell constituents, because coinjection of the wild-type *Xdsh* mRNA suppressed this phenotype. These findings suggest that the endogenous Xdsh is involved in the control of morphogenetic movements in the embryo. Surprisingly, Xdsh appears to regulate cell movements indirectly *via* secondary cell–cell interactions, because cells in which Xdsh signalling has been blocked inhibit cell movements in other cells which did not receive exogenous *Xdsh* mRNA. As Dsh is known to be involved in multiple signal transduction pathways, regulation of morphogenetic movements may be only one of its functions. Although these data indicate that the PDZ domain is a critical motif involved in the Xdsh signalling mechanism, different domains of Xdsh may participate in different signal transduction pathways. Further experiments are necessary to determine the molecular basis for Xdsh activity and to elucidate how Xdsh controls cell fates.

Materials and methods

Eggs and embryos

Eggs were obtained by injecting *Xenopus laevis* females with 700 U of human chorionic gonadotropin. *In vitro* fertilization and embryo culture were done in 0.1× MMR (1× MMR = 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES (pH 7.6), 0.1 mM EDTA) as described [54]. Staging was according to Nieuwkoop and Faber [55].

DNA constructs, in vitro *transcription and microinjections*

To generate efficiently translated mRNAs, the full-length *Xdsh* insert [15] was cloned into the *Eco*RI site of pCS2-MT$^+$ vector [56]. The *Xdd1* construct has an internal deletion of the DNA fragment corresponding to amino acids 301–381 of the Xdsh protein (Fig. 7b). The *Xdd2* construct encodes a carboxy-terminal deletion mutant that should retain the first 412 amino acids of the protein (from the total of 736 amino acids; Fig. 7b).

Capped synthetic RNAs were generated as described [57] by *in vitro* transcription of different plasmids using SP6 or T7 RNA polymerases. Templates were plasmids containing the entire coding sequence of *Xdsh*, *Xdd1*, *Xdd2*, *Xwnt8* [10,58], and *Xenopus* β-catenin [59] using Megascript RNA transcription system (Ambion). Embryos, incubated in 3 % Ficoll, 0.5× MMR, were injected with 10 nl of RNA solution in distilled water at the four- to eight-cell stage or with 5 nl of RNA solution at the 16–32-cell stage. After 2–3 h, the medium was changed to 0.1× MMR with 50 μg ml^{-1} of gentamycin for long-term culture. The death rate for the injected embryos was usually below 5 %. The prospective dorsal and ventral sides were determined by pigmentation differences in the early embryo [55]. For animal cap experiments, both blastomeres of two-cell embryos were injected in the animal pole region with 10 nl of mRNA solutions.

Explant culture, RNA isolation and northern analysis

Animal caps (approximately 1/5 of the size of the embryo) were isolated from the injected embryos at the midblastula stage (stage 8) as described previously [60], and were cultured in 0.6× MMR for 40 h at room temperature. Human recombinant activin A was used for stimulation of animal caps at 5 ng ml^{-1} [40]. Dorsal marginal zone explants were isolated at stage 10 [40], and cultured in 0.9× MMR until the control embryos reached stage 17.

Total RNA was extracted from cultured explants for northern analysis, when control embryos reached stage 11 as described [13]. Total RNA was separated in a 1 % formaldehyde-agarose gel using standard techniques [61]. RNA from ten animal caps or two embryos was loaded per lane. RNA was transferred to a GeneScreen nylon membrane (Dupont) with 20× SSPE and was sequentially hybridized with radiolabeled DNA or RNA probes [61]. Antisense RNA probes were prepared by *in vitro* transcription from plasmids containing *Xotx2* [62], the fibronectin gene [63], *Xlim1* [64], *goosecoid* [65] and *Xnr3* [66], using ^{32}P-UTP and SP6 or T7 RNA polymerase. DNA probes for *Xbra* [67] and *chordin* [68] were radiolabeled with ^{32}P-dCTP by Klenow enzyme using random hexamer primers [61]. After each hybridization cycle, the probe was stripped by boiling in distilled water. The same membrane was used for hybridization with all probes. Equal loading was controlled by the fibronectin antisense probe.

Lineage tracing, histology, whole-mount immunohistochemistry and in situ *hybridization*

Embryos were injected into both animal dorsal blastomeres at the eight-cell stage with 10 nl of a solution containing 1 ng of *Xdd1* mRNA and 0.2 ng of *β-gal* RNA in water. After two days of culturing in 0.1× MMR, embryos were fixed in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$ and 3.7 % formaldehyde [69]) for 30 min. To detect β-galactosidase activity, embryos were rinsed in PBS and incubated with 1 mg ml^{-1} X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6x3H_2O$, 2 mM $MgCl_2$ in PBS. The time of staining varied from 20 min to several hours at room temperature depending on the desired intensity.

For histology, embryos were fixed for an additional 2 h in MEMFA, dehydrated through ethanol-xylene series, embedded in Paraplast, and 8 μm sections were cut on a rotary microtome. Sections were stained with hematoxylin/eosin (Sigma) according to the manufacturers protocol. Whole-mount immunochemistry was carried out as described [69] using muscle-specific monoclonal antibodies 12/101 [70] and notochord-specific monoclonal antibodies MZ15 [71]. Whole-mount *in situ* hybridization was performed as described [72].

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References

1. Nusse R, Varmus HE: Wnt genes. *Cell* 1992, 69:1073–1087.
2. Parr BA, McMahon AP: ***Wnt*** genes and vertebrate development. *Curr Opin Genet Dev* 1994, 4:523–528.
3. Herman MA, Vassilieva LL, Horvitz HR, Shaw JE, Herman RK: The ***C. elegans*** gene ***lin-44***, which controls the polarity of certain asymmetric cell divisions, encodes a Wnt protein and acts cell nonautonomously. *Cell* 1995, 83:101–110.
4. Yang Y, Niswander L: Interaction between the signalling molecules WNT7A and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* 1995, 80:939–947.
5. Riddle RD, Ensini M, Nelson C, Tsuchida T, Jessell TM, Tabin C: Induction of the LIM homeobox gene ***Lmx1*** by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell* 1995, 83:631–640.
6. Parr BA, McMahon AP: Dorsalizing signal ***Wnt-7a*** required for normal polarity of D-V and A-P axes in mouse limb. *Nature* 1995, 374:350–353.
7. Stark K, Vainio S, Vassileva G, McMahon AP: Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* 1994, 372:679–683.
8. Herzlinger D, Qiao J, Cohen D, Ramakrishna N, Brown AMC: Induction of kidney epithelial morphogenesis by cells expressing Wnt-1. *Dev Biol* 1994, 166:815–818.
9. Munsterberg AE, Kitajewski J, Bumcrot DA, McMahon AP, Lassar AB: Combinatorial signalling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev* 1995, 9:2911–2922.
10. Sokol S, Christian JL, Moon RT, Melton DA: Injected Wnt RNA induces a complete body axis in ***Xenopus*** embryos. *Cell* 1991, 67:741–752.
11. Smith WC, Harland RM: Injected Xwnt8 RNA acts early in ***Xenopus*** embryos to promote formation of a vegetal dorsalizing center. *Cell* 1991, 67:753–765.
12. McGrew LL, Lai C-J, Moon RT: Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signalling cascade with ***noggin*** and ***follistatin.*** *Dev Biol* 1995, 172:337–342.
13. Itoh K, Tang TL, Neel BG, Sokol SY: Specific modulation of ectodermal cell fates in ***Xenopus*** embryos by glycogen synthase kinase. *Development* 1995, 121:3979–3988.
14. Perrimon N: The genetic basis of patterned baldness in ***Drosophila***. *Cell* 1994, 76:781–784.
15. Sokol SY, Klingensmith J, Perrimon N, Itoh K: Dorsalizing and neuralizing properties of Xdsh, a maternally expressed ***Xenopus*** homologue of ***dishevelled.*** *Development* 1995, 121:1637–1647.
16. Rothbacher U, Laurent MN, Blitz IL, Watabe T, Marsh JL, Cho KWY: Functional conservation of the Wnt signalling pathway revealed by ectopic expression of ***Drosophila*** dishevelled in ***Xenopus***. *Dev Biol* 1995, 170:717–721.
17. Funayama N, Fagotto F, McCrea P, Gumbiner BM: Embryonic axis induction by the armadillo repeat domain of β-catenin: evidence for intracellular signalling. *J Cell Biol* 1995, 128:959–968.
18. Karnovsky A, Klymkowsky MW: Anterior axis duplication in ***Xenopus*** induced by the over-expression of the cadherin-binding protein plakoglobin. *Proc Natl Acad Sci USA* 1995, 92:4522–4526.
19. He X, Saint-Jeannet J-P, Woodgett JR, Varmus HE, Dawid IB: Glycogen synthase kinase-3 and dorsoventral patterning in ***Xenopus*** embryos. *Nature* 1995, 374:617–622.
20. Pierce SB, Kimelman D: Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3. *Development* 1995, 121:755–765.
21. Dominguez I, Itoh K, Sokol SY: Role of glycogen synthase kinase 3β as a negative regulator of dorsoventral axis formation in ***Xenopus*** embryos. *Proc Natl Acad Sci USA* 1995, 92:8498–8502.
22. Heasman J, Crawford A, Goldstone K, Garner-Hamrick P, Gumbiner B, McCrea P, *et al.*: Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early ***Xenopus*** embryos. *Cell* 1994, 79:791–803.
23. Klingensmith J, Nusse R, Perrimon N: The ***Drosophila*** segment polarity gene ***dishevelled*** encodes a novel protein required for response to the ***wingless*** signal. *Genes Dev* 1994, 8:118–130.
24. Theisen H, Purcell J, Bennet M, Kansagara D, Syed A, Marsh JL: Dishevelled is required during Wingless signalling to establish both cell polarity and cell identity. *Development* 1994, 120:347–360.
25. Sussman DJ, Klingensmith J, Salinas P, Adams PS, Nusse R, Perrimon N: Isolation and characterization of a mouse homologue of the ***Drosophila*** dishevelled segment polarity gene. *Dev Biol* 1994, 166:73–86..
26. Bryant PJ, Watson K, Justice RW, Woods DF: Tumor suppressor genes encoding proteins required for cell interactions and signal transduction in ***Drosophila***. *Development* 1993, Suppl:239–249.
27. Ponting CP, Phillips C: DHR domains in syntrophins, neuronal NO synthases and other intracellular proteins. *Trends Biochem Sci* 1995, 20:102–103.
28. Yanagawa S van Leeuwen F, Wodarz A, Klingensmith J, Nusse R: The Dishevelled protein is modified by Wingless signalling in ***Drosophila.*** *Genes Dev* 1995, 9:1087– 1097.
29. Axelrod JD, Matsuno K, Artavanis-Tsakonas S, Perrimon N: Interaction between Wingless and Notch signalling pathways mediated by dishevelled. *Science* 1996, 271:1826–1832.
30. Hamburger V: *The Heritage of Experimental Embryology: Hans Spemann and the Organizer*. New York: Oxford University Press, 1988.
31. Gerhart J, Doniach T, Stewart R: Organizing the ***Xenopus*** organizer. In *Gastrulation.* Edited by Keller R, Clark WH Jr and Griffin F. New York: Plenum Press; 1991:57–77.
32. Stewart RM, Gerhart JC: The anterior extent of dorsal development of the ***Xenopus*** embryonic axis depends on the quality of organizer in the late blastula. *Development* 1991, 109:363–372.
33. Keller R: Early embryonic development of ***Xenopus*** laevis. *Methods Cell Biol* 1991, 36:61–113.
34. Gont LK, Fainsod A, Kim S-H, De Robertis EM: Overexpression of the homeobox gene Xnot-2 leads to notochord formation in ***Xenopus.*** *Dev Biol* 1996, 174:174–178.
35. Symes K, Smith JC: Gastrulation movements provide an early marker of mesoderm induction in ***Xenopus*** laevis. *Development* 1987, 101:339–349.
36. Manoukian AS, Yoffe KB, Wilder EL, Perrimon N: The ***porcupine*** gene is required for ***wingless*** autoregulation in ***Drosophila.*** *Development* 1995, 121:4037–4044.
37. Krasnow RE, Wong LL, Adler PN: ***dishevelled*** is a component of the ***frizzled*** signalling pathway in ***Drosophila.*** *Development* 1995, 121:4095–4102.
38. Amaya E, Stein PA, Musci TJ, Kirschner MW: FGF signalling in the early specification of mesoderm in ***Xenopus***. *Development* 1993, 118:477–487.
39. Niehrs C, Keller R, Cho KWY, De Robertis EM: The homeobox gene ***goosecoid*** controls cell migration in ***Xenopus*** embryos. *Cell* 1993,72:491–503.
40. Itoh K, Sokol SY: Heparan sulphate proteoglycans are required for mesoderm formation in ***Xenopus*** embryos. *Development* 1994, 120:2703–2711.
41. Moon RT, Campbell RM, Christian JL, McGrew LL, Shih J, Fraser S: ***Xwnt***-5A: a maternal ***Wnt*** that affects morphogenetic movements after overexpression in embryos of ***Xenopus*** laevis. *Development* 1993, 119:97–111.
42. Lue RA, Marfatia SM, Branton D, Chisti AH: Cloning and characterization of hdlg: the human homologue of the ***Drosophila*** discs large tumor suppressor binds to protein 4.1. *Proc Natl Acad Sci USA* 1994, 91:9818–9822.
43. Kim E, Niethammer M, Rothschild A, Jan YN, Sheng M: Clustering of Shaker-type K^+ channels by interaction with a family of membrane-associated guanylate kinase. *Nature* 1995, 378:85–88.
44. Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, *et al.*: Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α1-syntrophin mediated by PDZ domains. *Cell* 1996, 84:757–767.
45. Bhanot P, Brink M, Samos CH, Hsieh J-C, Wang Y, Macke JP, *et al.*: A new member of the ***frizzled*** family from ***Drosophila*** functions as a Wingless receptor. *Nature* 1996, 382:225–230.
46. Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P: Binding of GSK3β to the APC–β-Catenin complex and regulation of complex assembly. *Science* 1996, 272:1023–1026.
47. Matsumine AA, Ogai A, Senda T, Okumura N, Satoh K, Baeg G-H, *et al.*: Binding of APC to the human homolog of the ***Drosophila*** Discs large tumor suppressor protein. *Science* 1996, 272:1020–1023.
48. Du SJ, Purcell SM, Christian JL, McGrew LL, Moon RT: Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in ***Xenopus*** embryos. *Mol Cell Biol* 1995, 15:2625–2634.

49. Torres MA, Yang-Snyder JA, Purcell SM, DeMarais AA, McGrew LL, Moon RM: Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development. *J Cell Biol* 1996, 133:1123–1137.
50. Schweitzer R, Howes R, Smith R, Shilo BZ, Freeman M: Inhibition of ***Drosophila*** EGF receptor activation by the secreted protein Argos. *Nature* 1995, 376:699–702.
51. Chitnis A, Henrique D, Lewis J, Ish-Horowicz D, Kintner CR: Primary neurogenesis is ***Xenopus*** embryos is regulated by a homologue of the ***Drosophila*** neurogenic gene Delta. *Nature* 1995, 375:761–766.
52. Moos M Jr, Wang S, Krinks M: Anti-dorsalizing morphogenetic protein is a novel TGFβ homologue expressed in the Spemann organizer. *Development* 1995, 121:4293–4301.
53. Jones CM, Kuehn MR, Hogan BLM, Smith JC, Wright CVE: Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* 1995, 121:3651–3662.
54. Newport J, Kirschner M: A major developmental transition in early ***Xenopus*** embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* 1982, 30:675–686.
55. Nieuwkoop PD, Faber J: *Normal Table of Xenopus laevis (Daudin).* Amsterdam, Holland: North Holland; 1967.
56. Turner DL, Weintraub H: Expression of achaete-scute homologue 3 in ***Xenopus*** embryos converts ectodermal cells to a neural fate. *Genes Dev* 1994, 8:1434–1447.
57. Krieg PA, Melton DA: Functional messenger RNAs are produced by SP6 ***in vitro*** transcription of cloned cDNAs. *Nucleic Acids Res* 1984, 12:7057–7070.
58. Christian JL, McMahon JA, McMahon AP, Moon RT: ***Xwnt***8, a ***Xenopus Wnt***-1/***int***-1-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* 1991, 111:1045–1055.
59. McCrea PD, Turck CW, Gumbiner B: A homologue of the armadillo protein of ***Drosophila*** (plakoglobin) associated with E-cadherin. *Science* 1991, 254:1359–1361.
60. Sokol SY: Mesoderm formation in ***Xenopus*** ectodermal explants overexpressing ***Xwnt8***: evidence for a cooperating signal reaching the animal pole by gastrulation. *Development* 1993, 118:1335–1342.
61. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1989.
62. Pannese M, Polo C, Andreazzoli M, Vignali R, Kablar B, Barsacchi G, Boncinelli E: The ***Xenopus*** homologue of ***Otx2*** is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* 1995, 121:707–720.
63. Krieg PA, Melton DA: Developmental regulation of a gastrula specific gene injected into fertilized ***Xenopus*** eggs. *EMBO J* 1985, 4:3463–3471.
64. Taira M, Jamrich M, Good PJ, Dawid IB: The LIM domain-containing homeobox gene Xlim-1 is expressed specifically in the organizer region of ***Xenopus*** gastrula embryos. *Genes Dev* 1992, 6:356–366.
65. Blumberg B, Wright CVE, De Robertis EM, Cho KWY: Organizer-specific homeobox genes in ***Xenopus laevis*** embryos. *Science* 1991, 253:194–196.
66. Smith WC, McKendry R, Ribisi S Jr, Harland RM: A ***nodal-***related gene defines a physical and functional domain within the Spemann organizer. *Cell* 1995, 82:37–46.
67. Smith JC, Price BMJ, Green JBA, Weigel D, Herrmann BG: Expression of a ***Xenopus*** homologue of ***brachyury (T)*** is an immediate-early response to mesoderm induction. *Cell* 1991, 67:79–87.
68. Sasai Y, Lu B, Steinbeisser H, Geissert D, Gont LK, De Robertis EM: ***Xenopus chordin***: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 1994, 79:779–790.
69. Hemmati-Brivanlou A, Harland RM: Expression of an ***engrailed-***related protein is induced in the anterior neural ectoderm of early ***Xenopus*** embryos. *Development* 1989,106:611–617.
70. Kintner C, Brockes JP: Monoclonal antibodies identify blastemal cells derived from differentiating muscle in newt limb regeneration. *Nature* 1984, 308:67–69.
71. Smith JC, Watt FM: Biochemical specificity of notochord. *Differentiation* 1985, 29:109–115.
72. Hemmati-Brivanlou A, de la Torre JR, Holt C, Harland RM: Cephalic expression and molecular charaterization of ***Xenopus En-2***. *Development* 1991, 111:715–724.